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NUTRITION REVIEWS

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NUTRITION REVIEWS

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THE PHYSIOLOGICAL BASIS OF THIRST

The Internal Environment

Evaluation of the internal environment conditions which are critical in triggering thirst continues to present a challenging experimental problem. The "dry mouth" theory of fluid intake has all but been abandoned, while notions of a generalized dehydration state are somewhat lacking in specificity. At the turn of the century, André Mayer, on the basis of cryoscopic observations, proposed that the increase in blood osmotic pressure following water deprivation was the stimulus to thirst.

With experiments such as A. Gilman's (*Am. J. Physiol.* **120,** *323 (1937)*), it became clear that an increment in the effective osmotic pressure of extracellular fluid produces thirst. Gilman found that an intravenously administered, hypertonic sodium chloride solution elicited twice the water intake that a urea solution of the same tonicity did. The sodium chloride remains in the extracellular fluid space, while urea diffuses freely across cell membranes. J. H. Holmes and M. I. Gregersen (*Ibid.* **162,** *326 (1950)*) showed that it was indeed the increase in effective osmotic pressure that was critical, for the thirst effect was not specific to serum sodium increments but could be produced by other substances, such as sorbitol, which did not cross cell membranes.

At this time, E. B. Verney (*Proc. Roy. Soc. (London)* **B135,** *25 (1947)*), investigating the factors releasing antidiuretic hormone, also concluded that a small increment in the effective osmotic pressure of the blood supply to the supraoptic and paraventricular nuclei of the hypothalamus was an effective stimulus. Verney postulated the existence of osmoreceptors within these nuclei which detect changes in the effective osmotic pressure of the blood and regulate the release of antidiuretic hormone accordingly. Then B. Andersson (*Acta Physiol. Scandinav.* **28,** *188 (1953)*) demonstrated that this mechanism was related to water intake as well as renal conservation. Hydrated goats given minute intrahypothalamic injections of hypertonic sodium chloride drank copiously, while injections of isotonic sodium chloride produced no thirst.

Uniformity of osmotic pressure between intra- and extracellular space is regulated, not by the passage of ions, but by the exchange of water between these compartments. Any increase in extracellular fluid osmotic pressure, therefore, dehydrates the cellular space. The experiments discussed thus far cannot distinguish which of these internal events might be crucial as a stimulus to thirst: (1) increment in serum effective osmotic pressure, or (2) the consequent increase in intracellular osmotic pressure or shrinkage of cellular space. While it is not possible to dehydrate these compartments independently, it is possible to overhydrate the cells by depleting the extracellular fluid of its normal amount of electrolyte. By the technique of intraperitoneal dialysis, large quantities of sodium can be removed rapidly from the extracellular fluid. The resulting osmotic gradient produces a movement of water from the extracellular fluid into the cells.

If increased extracellular fluid effective osmotic pressure were the sole condition triggering thirst, we should expect decreases in water intake. If intracellular dehydration were the critical stimulus, we should also expect intake decreases. However, experiments on rabbits, rats, and dogs have shown increases in thirst following dialysis. This is probably the result of the decrease in extracellular fluid volume produced by dialysis. The question of the mechanism of

extracellular fluid volume reception and adjustment, which has become a central problem in the explanation of renal regulation, also has become a puzzle on the intake side of the ledger.

Much clinical evidence attests to the fact that salt depletion is associated with increased thirst. The thirst occasioned by salt losses from sweating in hot climates and which cannot be assuaged by drinking plain water, but is relieved by salt tablets, is well known. However, not all clinical views relating salt depletion and thirst are in agreement. H. L. Marriott (*Water and Salt Depletion, p. 41. Charles C Thomas, Springfield, Ill. (1950)*) states: "... there is no thirst; indeed, water is often repellent. I have seen men suffering from severe secondary dehydration (*i.e.*, salt loss) refuse water or spit it out." Such clearly contradictory reports doubtless indicate that the internal environment makes more distinctions about states of water-electrolyte depletion than do investigators and theorists.

While both increased serum effective osmotic pressure and decreased extracellular fluid volume are thirst stimuli, not all conditions giving rise to thirst can be simply reduced to these two alternatives. The excessive water intake of patients in a state of water intoxication, where serum osmolarity is low and extracellular fluid volume is increased, remains unexplained (V. Wynn and C. G. Rob, *Lancet* **I,** *587 (1954)*). Likewise, the precise stimulus for the massive fluid intake characteristic of diabetes insipidus remains obscure. Surgical destruction of the median eminence of the hypothalamus and the supraopticohypophyseal tract interferes with the elaboration of antidiuretic hormone. Logically enough, the resulting diuresis is followed by increased intake, but it is nonetheless disconcerting to learn that in diabetes insipidus there is no increment in serum sodium concentration, and extracellular fluid volume is expanded (S. M. Friedman *et al.*, *Am. J. Physiol.* **192,** *401 (1958)*). What, then, is the internal environment condition sustaining thirst in diabetes insipidus?

Hormonal Factors

F. C. Bartter and his associates have shown that the secretion of aldosterone can be controlled by extracellular fluid volume independent of concentration; increases in extracellular fluid volume decrease secretion, while decreases in volume increase secretion. It is possible that the extracellular fluid volume-regulating aspect of thirst is a function of aldosterone level. Although desoxycortiscosterone acetate administration does give rise to polydipsia, this has been interpreted as secondary to salt retention, rather than a direct hormonal sensitization of neural "drinking centers." Nor does pitressin seem to have any direct action on thirst mechanisms in either normal or diabetes insipidus animals.

The most promising evidence for the existence of a "thirst hormone" is the report by G. J. Gilbert (*Am. J. Physiol.* **191,** *243 (1957)*). Electrolytic destruction of the subcommissural area in rats produced a permanent adipsia resulting in death from dehydration. Injection of subcommissural area extract into normal rats resulted in a sharp but temporary depression in daily water intake.

Metabolic and Regulatory Polydipsia

Stimulation of limited areas in the hypothalamus by means of permanently implanted electrodes elicits polydipsia, while destruction of such areas produces an adipsic animal. Various hypothalamic lesions also have resulted in chronic polydipsia. There exists no crucial test distinguishing a metabolic polydipsia, which is simply secondary to a pre-existing polyuric state, from a regulatory polydipsia, wherein the large urine volume is a consequence of high fluid intake. (Exceptions to this are the cases of genetic renal defect which are refractory to pitressin treatment.)

The occurrence of regulatory or primary polydipsia is inferred from diverse clinical reports. It is assumed to originate from a central nervous defect which perhaps mimics the effect of electrical stimulation of a hypothalamic "thirst center." The low glomerular filtration rate, which results from the enforced dehyration of a diabetes insipidus patient, allows a quite efficient renal conservation of water, so that urine values are by no means an infallible guide to the evaluation of metabolic polydipsic states. Again, animals with different polydipsogenic hypothalamic lesions all yield a diabetes-insipidus picture when subjected to a hypertonic saline challenge.

However, in evaluating neurohypophyseal disfunction, J. F. Dingman, K. Benirschke and G. W. Thorn (*Am. J. Med.* **23,** *226 (1957)*) have obtained differential patterns of responsiveness to the antidiuretic stimuli, nicotine and hypertonic saline. The classification of such patterns holds the promise of an objective basis for distinguishing regulatory from metabolic polydipsia.

Central Nervous System: Sampling the Internal Environment

It has been shown that the blood-brain barrier is relatively permeable in certain delimited areas. Vital dyes such as trypan blue do not penetrate the barrier except at the following sites: supraoptic crest, intercolumnar tubercle, neurophyophysis and stalk, area postrema and the subcommissural area. It is undoubtedly significant that precisely these areas are intimately related to the control of body water. We have already mentioned Verney's supraoptic osmoreceptors; here, too, C. von Euler has recorded "osmo-potentials" following intracarotid injection of hypertonic saline. The neurohypophysis and stalk, in conjunction with the hypothalamus, elaborates, stores, and releases antidiuretic hormone.

Gilbert's experiments on the subcommissural area, previously described, have led him to postulate the elaboration of a thirst hormone in this area, and to consider the possibility that this region is the much sought "volume receptor." In recent work, he has shown histological changes is this area resulting from water deprivation. G. Farrell and his co-workers demonstrated that lesions and extracts of this area reduce and stimulate aldosterone secretion respectively. Finally, C. D. Clemente, J. Sutin and J. T. Silverstone (*Am. J. Physiol.* **188,** *193 (1957)*) recorded electrical changes just lateral to the area postrema in the medulla upon intravenous injection of hypertonic saline. These were interpreted as "osmo-potentials."

We suggest that these central areas, which are permeated by trypan blue, form a system which samples the effective osmotic pressure and perhaps the volume of the blood, and integrates this information into the adjustive actions of renal regulation and intake behavior.

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FOOD CONSUMPTION IN A HOT DESERT ENVIRONMENT

The caloric needs of military personnel carrying out light activities in a hot desert environment were found to be similar to caloric requirements under temperate conditions.

During the past five years the United States Army Medical Research and Nutrition Laboratory has been conducting nutritional surveys which included studies at five army camps and seven hospitals throughout the United States. Also studies have been performed under extreme subarctic conditions with troops performing heavy activities. Recently, C. F. Consolazio and associates (*Metabolism* **9,** *435 (1960)*)

attempted to establish the minimal energy requirements and also the nutritional status of troops performing light activities while living under extremely hot desert conditions. The studies were carried out at the Yuma Test Station, Yuma, Arizona in 1957.

Two groups of army personnel were used, one comprising the military police and the other the remaining headquarter enlisted personnel, who carried out light clerical activities. Food consumption at the army mess was measured for the entire group of individuals, but not on an individual basis. In addition, the quantity of food consumed from outside sources was computed. Weekly weighings determined any change in body weight. The food was prepared by regular army cooks and bakers who used the Master Menu as a guide for preparation, and the investigators did not change or interfere with either the type of food offered or the quantity.

The protein, fat and carbohydrate contents of the foods were determined by chemical means and the caloric content calculated from the diet composition. Samples were collected from five representative servings of each food item offered at each meal and these were subjected to chemical analysis. At the end of each meal all the edible foods left on the plates were combined, homogenized and analyzed. Thus, by knowing the total amount of food served, the amount returned and the number of persons eating at any given meal, it was possible to calculate how much fat, protein and carbohydrate an average individual ate.

In order to determine the amount of food eaten outside the mess, a questionnaire containing a list of all popular food items was given to each individual, who recorded the amount consumed each day. (Previous studies by this group have indicated that approximately 17 per cent of the total food consumption of military personnel is obtained outside the mess.) The protein, fat, carbohydrate and caloric content of these supplementary foods was obtained by using standard nutritional tables.

Three periods of food consumption were measured. For the first period lasting seven days, the food consumption of the headquarters and military police groups was measured separately. After one free day, the food intake during a six-day period again was measured for the separate groups. Finally, a six-day study period was set up in which the amount of food consumed for the combined groups was computed.

During each of the three study periods, the mean daily temperature averaged 93, 95 and 97°F with the maximum temperatures being 111, 109 and 109 for the same periods. The relative humidity for the three periods averaged 21 to 28 per cent. There were 238 persons studied in the headquarters personnel group during the first period and 214 during the second, while the military police group averaged 53 members during both periods. It should be noted that the average total food consumption from all sources for the headquarters personnel averaged 3999 and 4130 calories for the two study periods. There was no change in the average body weight of this group during the two periods.

The military police groups, on the other hand, had caloric intakes of 4237 and 4595 calories per man per day during the first and second periods, respectively. In spite of their larger caloric intake, however, this group lost the equivalent of 17 g. per day during the 14-day study period.

R. E. Johnson and R. M. Kark (*Science* **105,** *378 (1947)*) reported that the caloric requirement of soldiers was affected by environmental temperature, and they concluded that the energy requirement of soldiers varies inversely with the mean environmental temperature. Thus the Dietary Allowances of the Food and Agricultural Organization and the National Research Counsel now provide for climatic differences, recommending a five per cent increase or decrease in the energy requirement for every 10°C decrease or increase in a mean environmental temperature of 20°C.

By this standard, the personnel at Yuma, Arizona, should have a 10 per cent decrease

in energy requirement. Assuming, that these personnel have an average body weight of 73 kg. and that the mean outdoor temperature is 95°F, the suggested caloric intake should be 3069 calories per man per day, a value considerably lower than the intake actually determined. However, the present investigators believe that the elevation in the expected caloric intake results from the extra energy needed to produce sweat, an essential factor for maintaining the body temperature in this hot environment.

The daily energy expenditure was not computed for each of the groups. However, the military police group spent at least eight to nine hours outdoors in the heat when on duty and thus lost considerable amounts of sweat, which may explain in part their greater food consumption and loss of body weight. Also when off duty the personnel spent a fair amount of their time swimming or playing softball.

Normally in hot climates there is a tendency toward a reduced caloric intake, but in the present study the mess hall was air conditioned and considerably cooler than the outside environment. In addition to the cool atmosphere, large quantities of iced cantaloupe and watermelon, iced fruit juice and milk were available at each meal. Thus eating in the mess hall was always pleasurable. It is of interest that the high food consumption of the headquarters and military police group were in the same range as that of military personnel performing hard physical work at both a temperate environment (4265 calories per day) and in a subarctic environment (4163 calories per day). Thus this study indicates that there is no decrease in the required food intake of men caused by solar radiation and high environmental temperature.

It should be noted that about 40 per cent of the total calories consumed for both groups came from fat. This is in contrast to some studies which indicate that in tropical areas there is a low fat consumption. The present investigators believe that the increased fat consumption in their study was not due to environmental conditions but rather to food habits which did not change with the environmental temperature.

HORMONE EXCRETION, BEHAVIOR PATTERN AND CORONARY DISEASE

The urinary excretion of certain hormones was studied in an attempt to determine why a behavior pattern characterized by competitive activity and a sense of time urgency alters the incidence and intensity of blood and cardiovascular abnormalities.

Several studies have been published recently indicating that periods of occupational stress are associated with elevated blood cholesterol levels and, in some cases, increased rate of blood clotting (M. Friedman, R. H. Rosenman and V. Carroll, *Circulation* **17,** *852 (1958)*; P. T. Wertlake *et al., Proc. Soc. Exp. Biol. Med.* **97,** *163 (1958)*; C. B. Thomas and E. A. Murphy, *J. Chron. Dis.* **8,** *661 (1958)*; S. M. Grundy and A. C. Griffin, *Circulation* **19,** *496 (1959)*).

Rosenman and Friedman (*J. Am. Med. Assn.* **169,** *1286 (1959)*) have recently compared a group of men exhibiting chronic "excessive and competitive drive and an enhanced sense of time urgency" (pattern A) with a group exhibiting the opposite type of behavior (pattern B). It was found that the men with behavior pattern A, besides having a faster clotting time and higher serum cholesterol, also had about seven times higher incidence of clinical coronary artery disease and more than three times as much arcus senilis (a ring of degeneration about the corneal periphery). These investigators also found that females with behavior pattern A, when compared with females exhibiting pattern B behavior, had similar differences in blood and cardiovascular characteristics (*Circulation* **20,** *759 (1959)*).

Since these different types of individuals had similar diets and physical activity, Friedman, S. St. George, S. O. Byers and Rosenman (*J. Clin. Invest.* **39,** *758 (1960)*) studied the urinary excretion of possibly relevant hormones in an effort to determine how lipid changes might be induced by behavior pattern A.

Men between the ages of 35 and 50 were selected for this study on the basis of three main criteria. First, their occupations were associated with extreme competitive activity and preoccupation with deadlines; second, the individuals stated that they felt a sense of competitive drive and time urgency; and third, all men exhibited by their actions a severe sense of time urgency. Men with characteristics opposite to these were selected to represent pattern B behavior.

All subjects had normal blood pressure and were free of subjective symptoms of clinical coronary artery disease.

Urine was collected during working hours in order that the products of excretion would correspond to the time when the behavior pattern was most likely to be present. One urine sample was collected following bed rest. Each subject was asked to estimate his intensity of time urgency during the four days of urine collection.

The hormone assay included 17-ketosteroids, 17-hydroxycorticosteroids, 5-hydroxyindole, epinephrine and norepinephrine.

Records of dietary, tobacco, alcohol, work and exercise habits were made. Data were collected on serum cholesterol, blood clotting, and the incidence of arcus senilis and clinical coronary artery disease.

The number of working hours and the amount of physical activity were greater for the men in group A, but the exercise tended to be more concentrated on weekends. Alcohol and cigarette use was much greater in this group. Total calories, carbohydrate, protein and fat intakes were essentially similar in both groups.

Average serum cholesterol of group A (276 mg. per cent) was significantly higher than the average for group B (209 mg. per cent). Similarly, clotting time was significantly faster in group A (6.4 minutes) than in group B (7.2 minutes).

Arcus senilis was present in eight of the 12 men from group A and in none from group B. Four of the men in group A and none from group B had clinical but asymptomatic coronary artery disease.

The excretion of 17-hydroxycorticoids increased in both groups during working periods. The urinary excretion of 17 ketosteroids while working was about the same in both groups. The 5-hydroxyindole excretion did not differ between night or working hours or between groups.

Excretion of epinephrine increased markedly during working hours in both groups. Men of group A showed an increase of 86 per cent as compared with a 36 per cent increase for group B, but the difference was not statistically significant. On the other hand, men in group A showed an increase of 173 per cent in excretion of norepinephrine during working hours as compared with a 64 per cent increase for men of group B, and this difference was statistically significant.

In the opinion of the authors, two factors were responsible for the production of the behavior pattern A, namely, the intensity of the environment and the individual reaction to it. Modification of either factor could conceivably alter the biochemical response. They believe that any attempts to confirm these results should be characterized by rigid adherence to the proper conditions for the study of catecholamine excretion. In other words, if either the individual reaction or the special environmental conditions are lacking, the elevated norepinephrine excretion would probably not occur.

Whether a part-time discharge of excess catecholamines over a period of years could affect the pathogenesis of arterial disease is not known. Further studies correlating the

incidence of coronary artery disease with the presence of behavior pattern A would contribute to a better understanding of this problem. If atherosclerosis in experimental animals could be made more severe by administering norepinephrine, it would significantly implicate this compound in a causative rather than merely associative role.

METABOLIC CHANGES ACCOMPANYING LOW VITAMIN B_6 INTAKES

Changes occurring in college students on a low vitamin B_6 intake suggest that xanthurenic acid excretion after a tryptophan test dose, lymphocyte count and blood pyridoxine may indicate nutritional status for vitamin B_6.

The syndrome of a vitamin B_6 deficiency in man has not been clearly described. However, recent investigations, such as that by R. W. Vilter *et al.* (*J. Lab. Clin. Med.* **42,** *335* (*1953*)) of vitamin B_6 deficiency produced by feeding desoxypyridoxine and that by A. D. Hunt (*Am. J. Clin. Nutrition* **5,** *561* (*1957*)), have suggested that a deficiency of vitamin B_6 in man may be more prevalent than previously supposed. This assumption is partly based on the observation that a deficiency of pyridoxine in the human being may mimic changes occurring in deficiencies of other B vitamins. Unfortunately, the number of tests for vitamin B_6 deficiency in the human subject are limited and thus the changes in xanthurenic acid excretion following a test dose of tryptophan and the changes in blood vitamin B_6 concentration and in the lymphocyte count in the blood may be more suggestive than definitive in evaluating vitamin B_6 deficiency.

K. E. Cheslock and M. T. McCully (*J. Nutrition* **70,** *507* (*1960*)) have endeavored to follow the changes occurring in human subjects maintained for up to 52 days on a diet low in vitamin B_6. Their experiments were carried out on college students, seven women and one man, ages 18 to 20, who were fed a diet of natural foods found upon analysis to be low in vitamin B_6. The diets provided the women a daily average of 0.414 mg. of vitamin B_6 and 4.22 g. of nitrogen while the man received 0.50 mg. of vitamin B_6 and 5.75 g. of nitrogen per day.

Vitamins B_1, B_2, niacin, and 7 mg. of iron were added in order to insure that these dietary constituents were not inadequate. The diets supplied an adequate amount of energy and the subjects of this study showed very little weight change during the seven weeks of the experiment.

The only possible gross evidence of a vitamin B_6 deficiency was the complaint of excess fatigue on the part of some of the students. However, Cheslock and McCully observed that this complaint is a common one on the part of college students and may well be due to other factors.

Four weeks after the vitamin B_6-deficient diet was started, pyridoxine in the blood had dropped to zero and remained at this level until the end of the diet period. Administration of 100 mg. pyridoxine hydrochloride upon termination of the experimental diet resulted in a rapid increase in vitamin B_6 content of the blood to a level several times that found eight weeks later. There was a considerable variation in the blood content of vitamin B_6, ranging from 1.28 μg. per 100 ml. to 14.94 μg. per 100 ml. in a subject who had been suffering from a thymoma.

In five of the eight subjects, a 5 g. test dose of L-tryptophan resulted in an excretion of more than 30 mg. of xanthurenic acid per day, which is above the level at which a deficiency of vitamin B_6 may be thought to exist. Two other subjects exhibited increased excretion of xanthurenic acid, but at a lower level than 30 mg. The male subject showed very little change in xanthurenic acid excretion. Since he had received somewhat more vitamin B_6 than the women, it

was suggested by Cheslock and McCully that perhaps this amount more nearly met his requirements.

The blood studies were characterized by a decrease in lymphocyte count in five of the eight subjects. This decrease showed some significance and may have been the best evidence than a vitamin B_6 deficiency was developing. The subject with a thymoma did not show appreciable change in lymphocyte count.

The rapid drop of vitamin B_6 in the blood without other severe changes to indicate vitamin B_6 deficiency suggests that the human being may have a priority system respecting the use of pyridoxine within the body. The fact that, following tryptophan administration, three subjects did not experience a rise in xanthurenic acid excretion to a level suggestive of vitamin B_6 deficiency may mean that they were receiving sufficient vitamin B_6 to protect them from this metabolic change. Cheslock's earlier work with rats (*J. Nutrition* **65,** *53* (*1958*)) indicated that perhaps twice the normal requirement of pyridoxine must be fed before vitamin B_6 can be detected in the blood.

In evaluating the results presented by Cheslock and McCully it appears that young college women probably need more than 0.5 mg. of pyridoxine per day. However, it is also apparent that, at least for comparatively short periods of time, a diet containing less than this amount will not produce obvious signs of vitamin B_6 deficiency. Certainly this work emphasizes the need for more definitive methods of analysis to permit an accurate assessment of the metabolic changes occurring with low pyridoxine intakes. This is especially true if an effort is to be made to identify a subclinical deficiency of this vitamin in human subjects.

UTILIZATION OF FRUCTOSE BY WORKING MUSCLE

In a patient with an hereditary metabolic defect who developed muscular weakness on exercising, it was found that fructose could be utilized by the working muscle.

By studying the arterial-venous difference following an intravenous infusion of fructose, T. B. Van Itallie and associates (*Proc. Soc. Exp. Biol. Med.* **84,** *713* (*1953*)) and M. Miller *et al.* (*Yale J. Biol. Med.* **29,** *335* (*1956–57*)) obtained evidence to indicate that there was an uptake of fructose by the muscles of the forearm. This could be interpreted as utilization of fructose by these muscles.

Recently, C. M. Pearson and D. G. Rimer (*Proc. Soc. Exp. Biol. Med.* **100,** *671* (*1959*)) studied a patient who had a rare muscle disorder, previously described by B. McArdle (*Clin. Sci.* **10,** *13* (*1951*)). This individual was unable to continue moderate muscular activity for more than four minutes. Beyond this time there was rapid fatigue, followed by paralysis. It was noted that ischemically exercised forearm muscles became rapidly fatigued and there was no measurable increase in pyruvic or lactic acid levels in the venous blood draining the exercised arm. Normal individuals subjected to the same exercise had a fourfold rise in pyruvic and lactic acids in the venous blood coming from the tested arm. In an addendum, these investigators reported that the muscular defect in this individual was an absence of phosphorylase a and b.

For this study, the subject was exercised in the fasting state on a treadmill set at a 10 per cent grade and moving at the rate of four miles per hour. He received intravenously at different times normal saline, sodium bicarbonate, 10 per cent glucose or 10 per cent fructose solutions. The sugars were given at the rate of 0.73 g. per kg. per hour for 30 minutes prior to as well as throughout the exercise. In 20 test periods,

during which time no intravenous alimentation was given, it was found that the patient could walk on the treadmill for no longer than four minutes; the average duration of work was three minutes 20 seconds. Administration of saline or sodium bicarbonate did not improve the ability to perform.

The exercise tolerance, however, could be increased 20 times (the limit of the stress) by giving glucose intravenously during the exercise, the improved capacity occurring when the plasma glucose levels reached 160 mg. per cent and above. Upon administration of fructose, there was a similar improvement in the ability to exercise without fatigue so that walking could be continued for 60 minutes. In this case there was no significant increase in the plasma glucose level, but the fructose level rose to a maximum of 35 mg. per cent. When the infusion was stopped and the exercise continued it was possible for the patient to walk another 23 minutes, after which rapid fatigue and paralysis of the muscles of the legs developed. At this time the plasma fructose levels had fallen to 8 mg. per cent.

This study indicates rather clearly that fructose can increase the work capacity of muscles. It also suggests that the mechanism of action does not involve the conversion of fructose to glucose, with the latter serving as the direct source of energy, since there was no significant change in the plasma glucose levels during fructose administration. It was shown that, for glucose to be effective, the plasma glucose level must be about 160 mg. per cent, a value which did not occur following the administration of fructose.

It also appears from this study that fructose levels remain constant during exercise. Moreover, the entrance of fructose into the muscle occurs at a much lower level than for glucose, since at an extracellular fluid fructose level of about 10 mg. per cent muscular work could still be performed, whereas plasma glucose had to be increased to nearly twice the fasting level before work could be carried out by the muscle.

Another explanation for the ability of fructose to increase the work capacity of muscle is found in the abstract of Pearson, Rimer and W.F.N.M. Mommaerts (*Clin. Research* **7**, *298* (*1959*)). Using the same individual as reported earlier, they noted that in addition to glucose and fructose, lactate when given intravenously would increase the ability to exercise without fatigue. The possibility thus exists that lactate is the substance actually being utilized by the muscle, and hence the reason that fructose is effective at a lower blood level than glucose is that fructose is a much better lactate former. In the present study, however, one cannot establish whether fructose is used directly by the muscle or whether it is first converted to lactate so that the latter is the substance utilized.

Thus by observation of a young male subject with a metabolic disorder in which there appeared to be a failure to form lactic acid from glycogen under anaerobic conditions, it was found that the muscles can utilize fructose for energy purposes.

POSSIBILITY OF A NEW PITUITARY LIPEMIA-PRODUCING HORMONE

Injection of pituitary extract produces hyperlipemia in fed rabbits. A fraction has been prepared, free of the recognized pituitary hormones. Adrenocorticotrophin enhances its activity.

Not often is a new pituitary hormone demonstrated nowadays, but this may well have been accomplished in the studies of D. Rudman and others.

Rudman and F. Seidman became interested in "Lipemia in the rabbit following injection of pituitary extract" and their first paper, with this title, appeared in

1958 (*Proc. Soc. Exp. Biol. Med.* **99,** *146 (1958)*). Previous work of others had suggested that injection of pituitary substances leads to mobilization of depot fat and a rise in plasma non-esterified fatty acids, with liver fat deposition, increased fatty acid oxidation and formation of ketones.

Specifically, anterior pituitary extract and adrenotropic, growth or thyrotropic preparations had been demonstrated to increase liver fat; injection of growth hormone had been shown to cause an increase in plasma non-esterified fatty acids.

With these and related observations as a background, Rudman and Seidman studied the effect of pituitary extracts on serum lipids in the rabbit. Whole pituitary glands (lyophilized or acetone-desiccated) were extracted in the cold with one-tenth molar disodium phosphate. The rabbits were males weighing 2.5 to 4 kg., fed standard rabbit chow. They received a single subcutaneous injection of the pituitary extract and were not fasting except for blood sugar determinations.

One injection of extract from 100 mg. of lyophilized whole hog pituitary caused gross lipemia in 16 of 20 rabbits. Serum cholesterol and total lipids increased within six hours and rose sharply to a maximum in 18, usually returning to normal within 48 hours. The peak values shown for one rabbit were about four times the pre-injection level of serum cholesterol and seven times that for total lipids.

The increase in total serum lipids and in cholesterol was a straight-line function of the logarithm of the dose of pituitary extract. Plasma non-esterified fatty acids increased two- to threefold 18 hours after injection. Fasting blood sugar levels and glucose tolerance were not affected.

Extracts derived from lyophilized glands were three to four times more potent than those from acetone-desiccated glands. Hog, sheep, beef and human glands were all active. The activity of a single anterior lobe was about seven times greater than that of a single posterior lobe. Diffusion between lobes, as well as imperfect slaughterhouse separation, presumably accounts for the apparent activity of both lobes.

The six hormones of the anterior pituitary were individually tested in doses five or more times the quantities contained in the amount of pituitary extracted. No lipemia was detected at 18 hours, except in one of four rabbits receiving growth hormone.

Rudman and Seidman found their lipemia-producing material to have many of the physical characteristics of a protein. They concluded that they were dealing either with a distinct substance, or with a synergistic effect of two or more of the recognized pituitary hormones.

In the next publication of this group (except for two abstracts), Rudman, Seidman and M. B. Reid (*Proc. Soc. Exp. Biol. Med.* **103,** *315 (1960)*) have extended their studies, seeking to determine whether the lipemia-producing activity is due to synergism between recognized hormones or to a different and unrecognized substance. They have succeeded by fractionation in separating a lipemia-producing component from the other recognized pituitary hormones.

Lipemia production was measured by the change in total serum lipids of the rabbit 18 hours after subcutaneous injection of the test material. Other hormone activities were assayed by standard methods.

Since 15 rabbits showed an increase in total serum lipids of 50 $\pm$ 142 mg. per cent (after injection of 5 ml. of distilled water), the authors attributed significance only to increases of more than 380 mg. per cent ($p > 0.01$).

The six recognized anterior pituitary lobe hormones were separately tested. The dosages of adrenocorticotrophin (ACTH), growth hormone (GH) and thyroid-stimulating hormone (TSH) corresponded to the amount of each hormone present in 0.5 g. or more of desiccated gland. Prolactin, interstitial-cell stimulating (luteinizing) hormone (ICSH) and follicle-stimulating hormone (FSH) were

tested in 25 mg. amounts. None raised total serum lipids significantly. Injection of an alkaline extract of 60 mg. of desiccated hog pituitary gland produced an increase in serum total lipids of 1450 ± 205 mg. per cent. All 15 possible two-hormone combinations were tested, each hormone at half the dose used singly. Three pairs produced significant lipemia; these were ACTH plus TSH, prolactin or FSH. These experiments were repeated with purified TSH without effect, indicating that the synergism was between ACTH and some contaminating substance other than TSH. Purified prolactin and FSH were not similarly tested.

The authors next investigated whether synergism between ACTH and other components were responsible for the entire lipemia-producing activity of crude pituitary extract. Fractionation studies on acetone precipitates of a saline extract showed lipemia-producing activity only in the acetone 50 to 90 per cent fraction, and mainly in the 75 to 90 per cent fraction. Since the latter was previously known to be free from adrenocorticotrophin, growth hormone, prolactin and thyroid-stimulating hormone, it seemed clear that synergism of ACTH could account for only a part of the total lipemia-producing activity of the pituitary gland and that a substance different from ACTH, GH, TSH and prolactin was at work.

Rudman's group then devised a technique for concentrating the lipemia-producing substance. A saline extract of lyophilized intact hog pituitary glands was subjected to acetone fractionation in the cold. "Fraction G" yielded 15 mg. of material per gram of desiccated pituitary gland. Fraction G was then run through a column of a cation exchange resin and fractions were collected serially. Physiologic activity was found to be sharply concentrated in a few tubes containing about 30 per cent of the material put on the chromatographic column. The material in these tubes was pooled and precipitated by ammonium sulfate, dialyzed and lyophilized. 5 mg. of this material ("Fraction H") was obtained per gram of desiccated pituitary gland.

Three milligrams of Fraction H caused a 200 per cent increase in rabbit serum lipid level. Eighty-five per cent of the increase consisted of triglycerides, 5 per cent of cholesterol and 10 per cent of phospholipids. The action of Fraction H was intensified by injection with ACTH. Assays of Fraction H for the six recognized anterior and two posterior pituitary hormones showed none present in more than 0.8 per cent concentration. A mixture of the eight hormones representing their maximal content in up to 25 mg. of Fraction H was without significant effect upon rabbit serum lipid levels.

The authors recovered only 10 per cent of the original activity in their final Fraction H, whether because of loss, inactivation, or removal of synergistic hormone(s), *i.e.*, ACTH. They discuss the possible role of synergism between the known hormones and the existence of a hypothetical unknown "contaminant," *i.e.*, their own lipemia-producing substance. Their work gives no clue as to the mechanism of the synergistic effect of ACTH on lipid metabolism, which, they point out, may not even be adrenal-mediated.

Rudman, Seidman and Reid conclude that "lipemia-producing activity of crude hog pituitary extract is caused by the combined actions of (a) lipemia-producing component in Fraction H; (b) ACTH; and (c) substances present in commercial preparations of TSH, prolactin and FSH."

The authors appear to have demonstrated a lipemia-producing substance distinct from each of the nine recognized pituitary hormones. Vexing questions come up, however. Why should any other pituitary hormone (*i.e.*, ACTH) have any synergistic effect? This seems contrary to general experience in endocrinology, although not impossible. It is unlikely that the lipemia-producing factor itself could be a contaminant of the ACTH, even though this seems to be the explanation of the difference between TSH and purified TSH in combination with ACTH.

There is evidence that ACTH has a direct effect on adipose tissue, perhaps even remaining after corticotrophic action is destroyed. M. C. Schotz, G. M. C. Masson and I. H. Page have studied the effect *in vitro* of ACTH on the release of non-esterified fatty acids from adipose tissue of adrenalectomized rats (*Proc. Soc. Exp. Biol. Med.* **101,** *159* (*1959*)). They found that ACTH increased the release of fatty acids, that prior adrenalectomy decreased the process, and concluded that "ACTH may have a physiological role in fatty acid mobilization not mediated by adrenal cortical tissue."

How does Rudman's lipemia-producing effect differ from the effect of growth hormone, which also reduces fat stores, increases liver fat and causes ketosis? Injection of growth hormone into the dog or man produces a striking rise in fasting plasma non-esterified fatty acids, as reported by M. S. Raben and C. H. Hollenberg (*J. Clin. Invest.* **38,** *484* (*1959*)). This effect was suppressed by glucose or food or glucose and insulin, and the authors concluded that growth hormone influences the rate of fat mobilization in the postabsorptive period.

The essential difference in the two systems is that Rudman's group is working with the fed rabbit, in which the late effect of growth hormone on plasma non-esterified fatty acids is suppressed by feeding. However, in spite of the experimental differences between the two systems, there are some disconcerting similarities. Only much more work on the mechanisms involved will disclose whether these metabolic effects are in fact disparate. If they are, it seems strange. One might complicate matters further by asking whether Rudman's substance causes secondary changes in secretion of growth hormone or other substances concerned in fat mobilization.

The fat-mobilizing substance recently extracted from human urine by G. L. S. Pawan (*Biochem. J.* **74,** *29P* (*1960*)) may be similar or identical to Rudman's lipemia-producing substance. Pawan's material occurred in the urine of fasting human subjects and the pituitary was "necessary for its production." On hydrolysis, it yielded seven amino acids. In the mouse it increased fat mobilization and produced loss of body weight and reduction in blood sugar. Pawan concluded that it was neither growth hormone nor adrenocorticotrophic hormone.

Rudman and co-workers seem to have demonstrated a new pituitary hormone which produces lipemia. The mechanisms of the lipid-mobilizing effects of this substance and of growth hormone and ACTH are insufficiently defined at present for differences and similarities to be pointed out with any assurance. Likewise the relation of Rudman's to Pawan's substance is not established.

ALTERATION OF PROTEINURIA BY EXCESS FEEDING OF INDIVIDUAL AMINO ACIDS

An excess of poorly metabolized amino acids force fed to the rat produces an alteration of proteinuria. D-Serine also causes kidney tubule changes.

There has been much interest in the effects of prolonged feeding of an unbalanced diet and investigations have been carried out with both excesses and deficiencies of various dietary constituents. The importance of proteins and their constituent amino acids has been demonstrated by the changes which occur upon feeding excesses of individual amino acids.

Proteinuria in the rat has been shown to increase as the quantity of nitrogen in the diet increases. As long ago as 1926, T. E. Addis, M. Mackay and L. L. Mackay (*J. Biol. Chem.* **71,** *139* (*1926*)) reported on

changes in the kidney resulting from prolonged feeding of high levels of protein and cystine. This relationship between nitrogen intake and proteinuria in rats has led to investigations of the effect of added levels of individual amino acids upon proteinuria. R. W. F. Hardy and C. A. Baumann (*J. Nutrition* **69,** *429 (1959)*) demonstrated that increasing the level of dietary glycine resulted in a substantial decrease in proteinuria.

In a more recent study of the effects of a number of additional amino acids, Hardy, W. O. Tantengco and Baumann (*J. Nutrition* **70,** *438 (1960)*) observed that, while in general an increase of nitrogen in the diet of the rat increases proteinuria, the individual amino acids may have very different effects. The D and L forms of the amino acids also may have different actions, possibly depending upon the ease with which these forms are metabolized by the animal.

These investigators used mature rats maintained on a diet containing 20 per cent casein as the protein source. The rats were given by stomach tube 140 mg. doses of some 24 different forms of amino acids and then fasted for 24 hours. Urine collections were partitioned by paper electrophoresis and starch-gel electrophoresis, and this information was supplemented by chemical analysis for various urinary constituents.

Of particular interest were the results obtained following the administration of DL-serine, D-serine and L-serine. The losses of amino acids into urine accounted for only a small portion of the total administered dose, but in the case of DL- or D-serine, losses of 16 and 10 mg. of alpha-amino nitrogen per rat per day, respectively, were observed. This compares with only 2.6 mg. following the administration of L-serine. The increase in urea upon administration of many of the amino acids reflected the metabolism of the nitrogen from the administered amino acids. However, it is notable that this increase did not occur with DL or D-serine.

The increase in protein excretion in the urine upon administration by stomach tube of DL and D-serine has been observed by W. H. Fishman and C. Artom (*J. Biol. Chem.* **145,** *345 (1942)*), who also observed the sparing action of vitamin B_6 against the adverse effect of the DL-serine (*Proc. Soc. Exp. Biol. Med.* **57,** *239 (1944)*). Hardy, Tantengco and Baumann (*loc. cit.*) demonstrated that additions of pyridoxine at a level of 100 mg. per kg. of diet reduced the protein excretion produced by the administration of DL-serine, but they also observed that a 25 mg. per kg. addition of pyridoxine or the administration of an anti-metabolite, desoxypyridoxine, did not increase the protein excretion above that observed on the diet without added pyridoxine.

Experiments conducted by these investigators point up the inability of the rat to either metabolize or reabsorb from the tubule excretions large doses of D-serine. This was further confirmed by the observation that, following a single large dose of this amino acid, protein excretion in the urine remained high for at least three days.

The decreased urea excretion following administration of D-serine suggests an alteration in kidney function. Earlier work by R. P. Morehead, Fishman and Artom (*Am. J. Path.* **21,** *803 (1945)*) and by M. Wachstein (*Arch. Path.* **43,** *503 (1947)*) had indicated that the damage from this amino acid probably was localized in the distal region of the proximal tubule. Hardy, Tantengco and Baumann (*loc. cit.*), in extending their study to paper and starch-gel electrophoretic partitioning of the urine, observed that the protein excreted by rats fed excess DL-serine varied from the normal in that albumin became the major component and alpha-globulin was depressed.

Upon comparison with control animals, these results suggested that there is a much more pronounced reabsorption of albumins

and gamma-globulins than of either alpha-globulins or beta-globulins. While the D- and DL-serine produced the most marked variations from the controls, L-cysteine hydrochloride also increased urinary protein, but in the latter case, the normal distribution of proteins in the urine was found.

Hardy, Tantengco and Baumann have clearly demonstrated the effect upon the rat of a massive distortion of the normal balance of amino acids in the diet, especially notable upon administration of a form of the amino acid (in this case D-serine) which is not readily metabolized by the animal. It will be interesting to see whether a similar alteration is produced in other species.

EXPERIMENTAL ATHEROSCLEROSIS AND PYRIMIDINES

Rats fed cholesterol and cholic acid were used in experiments comparing the potencies of uracil, thiouracil and related compounds in producing thyroid changes, hypercholesteremia, azotemia and the beginnings of atherosclerosis.

Atherosclerosis in rats fed cholesterol and cholic acid is made worse by including thiouracil in the ration. In fact myocardial infarcts have been produced using this dietary modification. Naturally occurring pyrimidine derivatives have similar effects and uracil appears to be the most potent, according to L. C. Fillios, C. Naito, S. B. Andrus and A. M. Roach (*Am. J. Clin. Nutrition* **7,** *70 (1959)*).

Recently, Fillios, Naito, Andrus and Roach (*Circulation Res.* **8,** *71 (1960)*) compared the effects of uracil and thiouracil on experimental atherosclerosis in rats over a five-week period. Other related compounds were also investigated.

The atherogenic diet contained 20 per cent casein, 20 per cent hydrogenated cottonseed oil, 53.5 per cent sucrose, 1.5 per cent cholesterol, and 0.5 per cent cholic acid along with trace nutrients and salts.

In the first experiment, male rats averaging 260 g. were fed five different levels of thiouracil and compared with rats fed five different levels of uracil and with control rats fed only the atherogenic diet.

In the second experiment, males averaging 345 g. and females averaging 255 g. were compared. Each group was divided into subgroups fed either uracil, orotic acid, uracil plus orotic acid, thiouracil, or thiouracil plus orotic acid. Three other groups of males received supplements of (a) thymine plus uracil, (b) urea, or (c) aspartic acid.

Determination of serum urea levels of animals fed the various diets showed that feeding of uracil and thiouracil results in progressive elevation of urea with time. Although pathologic changes such as calcification in renal tubules and bladder stone formation occurred in the urinary tract, they did not appear to be related to the variation in urea concentration. Serum urea level also did not correlate with the extent of cardiovascular lipid deposition.

Serum cholesterol averaged 278 mg. per cent in control animals after five weeks. The addition of 0.25 per cent of uracil to the ration brought the average serum cholesterol to 555 mg. per cent, and addition of 1.0 per cent uracil gave the maximum average cholesterol of 704 mg. per cent. Thiouracil at 0.125 per cent of the diet produced an average serum cholesterol response of 717 mg. per cent, and the average maximum of 1100 mg. per cent cholesterol was reached with 0.25 mg. per cent of thiouracil. The authors state that in experiments not yet published, uracil at 1 per cent of the diet doubled the serum cholesterol values even on rations lacking cholesterol and cholic acid.

In the second experiment, urea, aspartic acid or orotic acid supplementation caused

no striking elevation of serum cholesterol. However, addition of 1 per cent uracil to the ration produced a marked rise in serum cholesterol, averaging 660 mg. per cent in males. This rise could be prevented by combining uracil and orotic acid. Addition of thymine, however, did not reverse the uracil effect. When thiouracil was fed, the average serum cholesterol of males rose to 1131 mg. per cent, but in males fed both thiouracil and orotic acid the average was 631 mg. per cent.

Female rats were more susceptible to hypercholesteremia on the atherogenic diets than males, but inclusion of orotic acid eliminated the sex difference. In fact, females fed orotic acid and uracil averaged 154 mg. per cent serum cholesterol contrasted with 1193 mg. per cent from feeding the uracil supplement only. Thiouracil gave an average serum cholesterol of 1882 mg. per cent in females, but feeding both thiouracil and orotic acid resulted in an average of only 456 mg. per cent.

Cardiovascular sudanophilia increased in the uracil-fed animals as the level of serum cholesterol increased and as the level of dietary uracil increased. Endocardial sudanophilia was higher in the thiouracil-fed animals and was uniform regardless of the dietary level of thiouracil. The aortic and mitral valves and intervening endocardium were involved. Varying degrees of aortic sudanophilia were observed, which were difficult to quantitate but were correlated with increased endocardial sudanophilia. None of the animals fed orotic acid showed any obvious aortic disease. The males supplemented with either urea or aspartic acid had less endocardial sudanophilia than the control animals.

Histologically, both uracil and thiouracil produce identical hyperplastic alteration of the thyroid differing only in degree. Comparisons of body weight, thyroid weight or serum cholesterol changes indicate that thiouracil is about 15 times as potent as uracil. Chemical analyses of the uracil certified its freedom from thiouracil contamination.

Hyperplasia of the thyroid was more marked in females fed uracil. On the other hand, females were more highly protected by the combined feeding of orotic acid and uracil as compared with males. Evidence of a protective action of orotic acid against thyroid hyperplasia due to feeding thiouracil was not definitive, either on the basis of organ weight or histological response. No definite histologic changes were seen when orotid acid was fed alone.

The goitrogenic action of uracil is unique among the naturally occurring pyrimidines. The compound could act in a manner similar to thiouracil or it could undergo conversion to a thiouracil-like compound in the organism. Orotic acid was used in this experiment because it is an intermediate in pyrimidine biosynthesis. Its action, according to the authors, might be related to its preferential position in the pathway of uridine-5-phosphate metabolism.

SELENIUM AND VITAMIN E DEFICIENCY IN LAMBS

Selenium administration protects against white muscle disease in vitamin E-deficient lambs and results in greater growth than does administration of vitamin E.

Evidence is accumulating that selenium is an essential trace element in animal nutrition and that it and vitamin E have a mutually sparing effect in a number of experimental situations. The subject has been reviewed at length in this journal (*Nutrition Reviews* **16,** *149, 174, 319 (1958)*). As pointed out, it previously had not been known that selenium was biologically essential except in the case of certain plants and in one bacterial enzyme system. However, work discussed in that review suggested that selenium is an

essential trace element in higher animals. At least two conditions were at that time preventable by either vitamin E or selenium: dietary liver necrosis in the rat and exudative diathesis (and "enlarged hock disorder") in the chick.

K. Schwarz, who has done considerable research on these dietary interrelationships, has recently reviewed "Factor 3, selenium and vitamin E" (*Nutrition Reviews* **18,** *193 (1960)*). He points out that selenium is an essential dietary constituent, and neither takes the place of vitamin E nor substitutes for it. He distinguishes diseases caused purely by vitamin E deficiency from others caused purely by Factor 3-selenium deficiency and still others caused by a simultaneous lack of both factors.

White muscle disease of lambs and calves evidently falls in the last group. Although there is evidence that this represents a vitamin E deficiency, selenium has also been reported to be protective (O. H. Muth *et al., Am. J. Vet. Res.* **20,** *231 (1959)*; (*Nutrition Reviews* **17,** *171 (1959)*).

In a more recent paper, J. E. Oldfield, Muth and J. R. Schubert (*Proc. Soc. Exp. Biol. Med.* **103,** *799 (1960)*) have shown that selenium or vitamin E is protective against white muscle disease of lambs and that selenium appears to be essential for growth.

The authors studied 48 pregnant ewes divided into four groups. The basal ration, presumably deficient in vitamin E and selenium, consisted of four pounds of Ladino clover hay and one-quarter pound of ground oats per head daily. The 20 lambs born of this control group showed an average six weeks' gain of 15.32 pounds; 16 of the 20 had gross lesions of white muscle disease. Ewes of lots two and three ate the same ration, but lot-two lambs were given 1.40 mg. selenium (as sodium selenite) intramuscularly (0.28 mg. at one day and 1.12 mg. at 14 days of age) while lot-three lambs received 1,000 IU vitamin E by mouth at one day and four days of age. Lot-four ewes were given the basal ration plus 0.1 p.p.m. selenium (as sodium selenite). These diet treatments extended from the third month of gestation until the lambs were six weeks old.

All supplements protected completely against symptoms and gross lesions of white muscle disease in the lambs. However, pronounced growth differences were manifest. The six weeks' gain of the vitamin E-supplemented lambs (17.33 pounds) was not significantly greater than that of the unsupplemented group with white muscle disease (15.32 pounds). On the other hand, lambs given selenium averaged a gain of 20.84 pounds, while the increase in those from selenium-fed ewes averaged 27.15 pounds. The authors infer that selenium in the neonatal period, while correcting white muscle disease, is not available early enough to allow the full growth exhibited by lambs of selenium-treated dams. They conclude that these growth responses "appear to be further confirmation of the role of selenium as an essential nutrient, per se, necessary for normal growth even in absence of WMD [white muscle disease] lesions."

The authors do not discuss the content of selenium, vitamin E or oxidants and antioxidants in the ration they used. History warns of potential errors in interpretation in this area. In addition, the material was not studied microscopically, so that one cannot be sure that the muscle lesions were comparable in the various treated groups. Perhaps they differed in severity. Possibly varying the doses of vitamin E and selenium would show the apparent difference to be a dosage effect.

One would also like to know the effects on growth of selenium plus vitamin E at various dosages; are they additive or not? Further observations on this point might strengthen the conclusion that selenium is in itself essential for growth. This parameter of nutritional research is a very non-specific term to the experimental pathologist, to whom impaired growth encompasses most known diseases.

Oldfield, Muth and Schubert point out that selenium compounds have been inef-

fective in at least two vitamin E deficiency syndromes: muscular dystrophy in rabbits and fetal resorption in rats. Nonetheless, they have supplied one additional example of the mutually sparing effect of selenium and vitamin E, while strengthening the opinion that their biologic effects are not identical.

EFFECTS OF DIETS DEVOID OF VALINE AND LYSINE ON YOUNG RATS

Force feeding of a diet devoid of valine and lysine produces a pathology in young rats similar to that of protein deficiency, with biochemical changes resembling those of kwashiorkor.

The changes produced in human beings suffering from kwashiorkor indicate the urgent need for a better understanding of the mechanisms involved in the metabolism of dietary constituents, especially of protein and its amino acids. In a continuing series of experiments, H. Sidransky and co-workers have been investigating the morphological and biochemical changes occurring in young rats fed diets deficient in single amino acids. The very rapid development of changes in young rats force fed diets devoid of specific amino acids has provided a means of studying lesions which resemble those of kwashiorkor.

It has been particularly interesting that the changes resulting from feeding diets deficient in threonine, methionine and histidine have been remarkably similar (Sidransky and E. Farber, *Arch. Path.* **66,** *119, 135 (1958)*). In an extension of this program, Sidransky and T. Baba (*J. Nutrition* **70,** *463 (1960)*) have described the changes occurring in young rats fed diets devoid of valine and lysine. These changes resembled those resulting from diets deficient in threonine, methionine and histidine, and suggest that the deficiencies of many essential amino acids may produce changes which reflect an overall protein deficiency rather than a deficiency of a specific amino acid. For this reason tissue alterations differ very little.

On the other hand, dissimilar changes as a result of feeding diets deficient in individual amino acids suggest that some of the pathology which develops may be related to specific functions of individual amino acids. When Sidransky and Baba force fed a diet devoid of valine or lysine to young rats weighing approximately 72 g. at the start of their experiments drastic changes were observed by the end of five or six days. A high rate of mortality in these rats led to an investigation of changes occurring by the end of three days. They concluded that many of the changes observed were well advanced by the end of three days of force feeding.

While rats on the control diet gained weight and showed no pathological changes, the animals fed the deficient diets lost weight even though the amounts fed were the same.

When fed ad libitum the valine- and lysine-devoid diets were poorly consumed and the animals lost weight, but they did not exhibit the changes observed in the animals force fed the diets devoid of valine or lysine.

The diet devoid of valine produced much more marked changes than that devoid of lysine. The hair of the valine-devoid rats soon became rough and shaggy and the animals grew listless and weak, with as many as 50 per cent dying after the first three days.

Of particular note were the liver changes. The total liver lipid content was significantly greater in the valine-devoid group than in the control group. The composition of the lipid was changed with a slight increase in cholesterol and a large increase in neutral fat. In the valine-devoid group there was also an increase in the liver glycogen content. Interestingly enough, the liver protein analysis was essentially the same for the amino acid-devoid groups and the controls.

In the valine-devoid group there was a sharp decrease in the weight of the skeletal muscle (as exemplified by the gastrocnemius) accompanied by a decrease in protein content, but only a weight decrease in the lysine-devoid group.

These continuing investigations into the role of specific amino acids and the possible relationship of protein and amino acid deficiencies to biochemical changes in animals which resemble those occurring in kwashiorkor, have certainly demonstrated that a deficiency of a single amino acid may be reflected as a protein deficiency with the major changes attributable to the low protein. Other biochemical changes, however, may be associated with the lack of a specific amino acid. By their force-feeding technique, these investigators have re-emphasized the role of amino acid balance and permitted an accentuation of morphologic and biochemical changes which may not appear with ad libitum feeding.

VITAMIN K DEFICIENCY AND COPROPHAGY IN THE RAT

Vitamin K deficiency, as manifested by increased prothrombin time, is uniformly induced in the rat by dietary deficiency of this vitamin if coprophagy is prevented.

The failure to elicit manifestations of certain vitamin deficiencies in rats fed a synthetic diet deficient in that particular vitamin has been due, in many instances, to coprophagy since intestinal bacteria are capable of synthesizing many vitamins. The rat is quite resistant to the dietary induction of vitamin K deficiency, although occasional instances have been reported (J. D. Greaves, *Am. J. Physiol.* **125,** *429 (1939)*). This resistance may be an effect of the great tendency of this animal to eat its feces. This possibility is explored in a recent report (R. H. Barnes and G. Fiala, *J. Nutrition* **68,** *603 (1959)*).

These investigators fed male weanling rats a synthetic diet deficient in vitamin K and containing 1 per cent stripped corn oil as the sole source of fat. It was not possible to induce vitamin K deficiency with a diet containing 15 per cent hydrogenated vegetable oil, apparently because of the small amount of vitamin K contained in this preparation. Coprophagy was prevented by a small plastic cup covering the anus (Barnes *et al.*, *J. Nutrition* **63,** *489 (1957)*). One-stage prothrombin times were determined on whole blood from the tail. Results of such determinations checked well with similar determinations using oxalated plasma from heart blood.

Prothrombin times increased strikingly by the third to sixth weeks and then, peculiarly, decreased toward the normal level. Animals allowed access to their feces did not develop increased prothrombin times. Since several of the animals died during the period when the prothrombin time was greatly elevated, the possibility existed that the ultimate decline in prothrombin time was due to the selective survival of rats that showed little or no elevation.

The data were recalculated for only the animals which survived the vitamin K deficiency until the prothrombin time was again at a low level. Still the same typical curve with an elevation and a subsequent fall toward normal resulted, indicating that the terminal decrease was not due to selective survival of resistant animals. As the authors point out, this type of prothrombin time response seems to be typical of other vitamin K-deficient animals (T. D. Luckey, J. R. Pleasants and J. A. Reyniers, *J. Nutrition* **55,** *105 (1955)*).

They also note that prothrombin time alone, as measured by the one-stage technique, does not measure the entire defect, since vitamin K deficiency decreases plasma

thromboplastin component (Factor IX) and proconvertin (Stable Factor) as well (R. L. Naeye, *Proc. Soc. Exp. Biol. Med.* **91,** *101 (1956)*; L. M. Fisher, G. J. Millar and L. B. Jacques, *Canad. J. Biochem. Physiol.* **34,** *1039 (1956)*). Indeed, it has been questioned by some whether abnormalities in clotting mechanism alone could entirely explain the hemorrhagic phenomena which result (S. B. Wolbach and O. A. Bessey, *Physiol. Rev.* **22,** *233 (1942)*). However, most of the animals that died did so during the period of greatest elevation of prothrombin time, although some died after it had decreased to a lower level.

The influence of vitamin K storage on the development of deficiency was studied by feeding animals the deficient diet while still allowing them access to their feces. At intervals up to 12 weeks, fecal collection cups were attached to groups of animals and prothrombin times determined. After three weeks on the deficient diet, there was an immediate increase in prothrombin time as soon as the animals were prevented from eating feces. However, during the first three weeks on the diet there was a lag period of one to two weeks after coprophagy was prevented before prothrombin times increased. This apparently indicates a storage of vitamin K, which is depleted after about three weeks on the deficient diet.

In another aspect of the study, two rats were caged together and fed the deficient diet. One was prevented from eating feces by the attachment of the plastic cup, whereas the second animal was allowed access to its own feces. Evidence of vitamin K deficiency, as manifested by an increased prothrombin time, occurred only in the animal with the plastic cup attached. However, this animal also probably managed to eat some feces from his cagemate since the prothrombin time increase was not as great as in control animals which were caged alone and which were prevented from eating feces.

Menadione in the diet, in amounts from 0.1 to 10.0 mg. per 100 g. of diet, prevented the development of vitamin K deficiency whether or not coprophagy was prevented. As little as 1.0 mg. of either menadione or vitamin K_1 per 100 g. body weight given by stomach tube caused the prothrombin time to return to normal within 18 hours. Vitamin K_1 in doses of 0.5 mg. per 100 g. body weight had the same effect, but 0.1 mg. per 100 g. body weight was ineffective. It would seem that menadione is as effective as vitamin K_1 in reducing the prothrombin time in vitamin K-deficient rats, and that the minimal effective dose is somewhere between 0.1 and 0.5 mg. per 100 g. body weight.

There was a great variability between individual groups of animals in the spontaneous development of dietary vitamin K deficiency. Although only 19 animals developed signs of deficiency out of 84 rats fed the vitamin K-deficient diet but not prevented from eating feces, 16 of the 19 were from one study with 20 animals. In four similar experiments employing 39 rats no instance of deficiency was noted, and in two other groups with 25 rats three animals showed increased prothrombin times. The authors wisely conclude that the percentage incidence of the development of spontaneous hypoprothrombinemia would be of limited usefulness in view of this great variability. Much of this variability may be due to variations in the tendency of individual rats to eat feces.

It has been presumed that the relative difficulty in producing vitamin K deficiency in rats by dietary restriction is due to the synthesis of vitamin K by microorganisms in the intestine (*Nutrition Reviews* **3,** *35, 48 (1945)*), and that the most effective means is to supplement the diet deficient in vitamin K with an antibiotic agent effective against intestinal bacteria (*Ibid.* **1,** *35 (1942)*; A. Kornberg, F. S. Daft and W. H. Sebrell, *Pub. Health Reports* **59,** *832 (1944)*). *Escherichia coli* have been shown to possess a great ability to synthesize this vitamin, and the intestinal absorption of vitamin K produced by colon bacteria is thought to be sufficient

to supply the needs of most mammals (*Nutrition Reviews* **4,** *324 (1946)*).

However, the ability of the rat to absorb vitamin K from the intestine is questioned by the results reported in the present study. Indeed, the rat may have to eat its feces in order to get some of the vitamins synthesized in its intestine. The relationship of the rise and fall of the prothrombin time in vitamin K-deficient rats to intestinal synthesis and absorption is not known, although a similar curve has been found for vitamin K-deficient, germ-free chicks (Luckey, Pleasants and Reyniers, *loc. cit.*). A somewhat puzzling note is added by the report of the inability to produce dietary vitamin K deficiency in germ-free rats (Luckey *et al.*, *J. Nutrition* **57,** *169 (1955)*) and the conclusion that vitamin K is not required by the rat (*Nutrition Reviews* **14,** *116 (1956)*). It is possible that the diet used contained enough vitamin K to prevent induction of deficiency.

The ultimate mode of action of vitamin K is unknown at the present time. The factors in the clotting mechanism known to be affected by vitamin K deficiency are produced in the liver. Vitamin K may be concerned with the coupling of phosphorylation and oxidation in this organ (*Nutrition Reviews* **14,** *211 (1956)*; J. P. Green, E. Sondergaard and H. Dam, *Biochem. Biophys. Acta* **19,** *182 (1956)*). Striking histologic abnormalities have not been noted in the liver or other organs except for hemorrhages (K. M. Endicott, Kornberg and Daft, *Pub. Health Reports* **59,** *49 (1944)*; A. Ferraro and L. Roizin, *J. Neuropath. Exp. Neurol.* **2,** *392 (1943)*).

Electron microscopic examination of the liver and of capillaries in all parts of the body might be particularly informative; the former because it is the probable locus of action of vitamin K and the latter because alterations in capillary walls may be partially responsible for hemorrhagic phenomena.

This study re-emphasizes the fact that vitamin K is necessary in synthetic diets for the rat. Bacterial synthesis of vitamin K in the rat with absorption from the intestine is apparently not enough to meet the demands of the rat on a deficient diet. Coprophagy then enables the rat to utilize such material. This is a variable which must be considered in all studies attempting to induce dietary vitamin deficiency. This report affords another example of consistent dietary induction of a vitamin deficiency only after coprophagy is prevented.

EFFECT OF INSULIN AND GLUCOSE ON TISSUE MAGNESIUM

Administration of glucose and insulin to rabbits accelerates the uptake of radioactive magnesium^{28} in many tissues and increases the magnesium content of skeletal muscle and heart.

A number of intracellular constituents accumulate when glycogen is formed, either because of increased cell volume or because of chemical combination. Thus the administration of glucose and insulin, by increasing intracellular glycogen, causes substantial shifts of potassium, the principal intracellular cation, into the cell. The physiologic consequences of potassium derangements are particularly noticeable in cardiac and skeletal muscle where membrane electrical potentials or potassium content are significantly altered, for example in hypokalemia and in familial periodic paralysis.

One might expect that the movement of other cell electrolytes would follow that of glycogen and potassium. In the case of magnesium, because of its largely intracellular position, one might anticipate physiological results of some consequence.

Recent developments in our knowledge of magnesium metabolism have been reviewed in this journal (*Nutrition Reviews* **18,** *72, 101, 200 (1960)*. Magnesium depletion

occurs in certain patients with alcoholism or electrolyte imbalance, especially in patients treated with magnesium-free parenteral fluids for long periods. The resulting syndrome is quite like that of low-calcium tetany. Because magnesium and potassium are both predominantly intracellular ions, there are a number of similarities in their metabolism.

J. K. Aikawa has studied the "Effect of glucose and insulin on magnesium metabolism in rabbits", using radioactive magnesium[28] (*Proc. Soc. Exp. Biol. Med.* **103,** *363 (1960)*) and has found increased magnesium in heart and skeletal muscle.

Rabbits were fed a stock pellet ration. The author injected tracer doses of magnesium[28] in 1 to 2 mEq of stable magnesium chloride intravenously into each rabbit, followed by insulin, glucose, or both. Relative activity was calculated as counts per minute per gram of tissue divided by counts per minute per ml. of plasma and determined for various tissues, as was the magnesium content. The animals were sacrificed four hours after the first injection.

One group received only a tracer dose of magnesium[28]. The resulting specific activities were: bone, 12.9; kidney, 11.0; heart, 5.9; liver, 5.1; appendix, 3.9; skin, 1.25; muscle, 0.5. Another group received magnesium[28] plus subcutaneous insulin and dextrose. Apparently two different dosage combinations were used in each group and the results averaged. Relative magnesium activity increased significantly above the values in the control group for all tissues studied; it more than doubled in heart and skeletal muscle. Significant increases in magnesium content occurred in muscle (from 21.5 to 28.6 mEq per kg. of wet tissue) and in heart (from 13.0 to 23.5 mEq per kg.). The blood glucose value fell from 82.3 to 69.9 mg. per cent, but this was not considered significant (P was not <0.01).

When magnesium was followed by dextrose alone there was an increase in specific activity of all tissues, considered significant only in the case of heart. Significant increases in magnesium content were found in both skeletal muscle and heart. Magnesium followed by insulin led to increased activity of all tissues, but the standard error of the mean was very large and the increases were not significant. There was a slight significant increase in heart magnesium content.

Aikawa points out that magnesium[28] is concentrated slowly in bone of man and dogs. In the rabbits, however, it was taken up most rapidly by bone, which had a magnesium content 13 times that of muscle, the tissue with the next highest content.

Although the dose of magnesium (1 to 2 mEq) was necessarily large because of the low specific activity of the material, serum magnesium levels were unchanged four hours after injection. Tissue levels were not compared but might well have also manifested little change.

The author mentions various reports relating magnesium, blood sugar and insulin and discusses the effect of magnesium on glygogenesis, on phosphatases and on adenosine triphosphate without arriving at a definite hypothesis. He concludes that insulin and glucose lead to deposition of magnesium in heart and skeletal muscle, and infers that "metabolism of magnesium is intimately related to that of carbohydrate and that the turnover of magnesium in tissues is regulated by insulin and dextrose."

The author's data leave some questions in the reader's mind. All three experimental groups consist of eight rabbits each, with four animals on each of two different dosage schemes. The results of the two subgroups are averaged in each group and the reader wonders why. The standard error of the mean for specific activities is as much as ten times higher in the magnesium plus insulin group as in the others; we are not told whether the variation in dosages or other factors are responsible for this. It is

difficult to appraise the method used for tissue magnesium determinations; it is defined only by a primary microfilm reference source. However, one infers that the method is satisfactory since the results are consistent with what one would expect from previous work on magnesium metabolism and similar work on potassium.

Tissue glycogen and potassium contents were not determined; it would be desirable to compare these values with magnesium content.

In the absence of a detailed hypothesis of the interactions of magnesium, it seems simplest to explain Aikawa's results by suggesting that insulin and glucose cause increased intracellular glycogen deposition, increasing the fluid content of the cell and, with this, of the intracellular electrolytes including magnesium.

VITAMIN B_6 DEFICIENCY AND PREGNANCY IN THE RAT

Vitamin B_6 deficiency in the rat results in impaired production of red blood cells and hemoglobin. This defect is potentiated by pregnancy and may reflect a generalized defect in protein metabolism.

Vitamin B_6 (pyridoxine, pyridoxal and pyridoxamine) has been implicated as being necessary in both fat metabolism, especially essential fatty acids, and in protein metabolism (*Nutrition Reviews* **12,** *186* (*1954*)). As a precursor to pyridoxal phosphate it functions as a coenzyme in several transaminating and decarboxylating reactions. An adequate supply of this vitamin is necessary for maintenance of normal protein metabolism and growth. Thus the effects of deficiency should be especially acute during periods of maximal protein demand for tissue growth, such as during pregnancy. Alterations in plasma protein, hemoglobin, and hematocrit in the pregnant rat have recently been reported (R.L. Pike and M.L. Brown., *J. Nutrition* **68,** *551* (*1959*)).

These investigators placed female rats weighing about 200 g. in eight groups. One group of nonpregnant rats was fed an adequate basal synthetic diet supplemented with 0.8 mg. pyridoxine per 100 g. diet and another nonpregnant group was fed the basal diet containing 4 mg. deoxypyridoxine per 100 g. diet. Two groups of pregnant rats were fed similar diets. Other animals were fed the deoxypridoxine-supplemented diet for one to four weeks prior to mating to test the effect of depletion of vitamin B_6 stores on pregnancy. Subsequent to mating, one group of the latter animals was continued on the deoxypyridoxine-containing diet while the remainder were fed the diet supplemented with pyridoxine.

Nonpregnant controls for the latter two groups underwent a uniform depletion period of 21 days and were then divided into two groups, one of which was continued on the vitamin B_6-deficient diet and the other fed the vitamin B_6-supplemented diet. Blood was collected on the first, eighth, fifteenth, and twenty-first days of pregnancy. Specific gravities of whole blood and plasma were determined. Hematocrit, hemoglobin, and plasma protein levels were calculated from the specific gravity values.

During a three-week period, vitamin B_6 deficiency in nonpregnant animals resulted in a steadily increasing plasma protein level (a total increase of about 7 per cent of the initial value) and an early rise in hemoglobin and hematocrit with a subsequent plateau when compared to the nonpregnant animals supplemented with vitamin B_6. A somewhat similar response occurred in the deficient and supplemented pregnant animals during the first two weeks. During this period the plasma protein level in the deficient animals rose slightly, but then fell precipitiously during the third week (a total decrease of about 11 per cent of the

initial value), as it did in the pregnant supplemented group.

Prior vitamin B_6 depletion prevented pregnancies from reaching term in those animals continued on the deoxypyridoxine-supplemented diet; in half of the group implantation did not occur and in the other half the fetuses were resorbed prior to the third week. In the depleted group fed a pyridoxine-supplemented diet after mating, the response was closely similar to that in non-depleted, non-deficient pregnant animals. Rats continued on the deficient diet (no pregnancies reaching term) showed a response intermediate between pregnant and non-pregnant deficient animals.

Pregnancy affected both depleted and non-depleted, supplemented and deficient animals in similar manner. There was a steady decline in these values during the entire three weeks of pregnancy (a total decrease of about 20 per cent of the initial value). However, the decrease in these values in pregnant deficient animals (both depleted and non-depleted) occurred earlier and was quantitatively greater than that in the pregnant, supplemented animals. Since nonpregnant, deficient animals showed a similar response, it seemed that the diet and pregnancy had a synergistic action in lowering the hemoglobin and hematocrit levels.

The authors believe that these changes in plasma protein, hematocrit, and hemoglobin levels reflect altered ability of the organism to form plasma proteins, red blood cells and hemoglobin. However, as they point out, many of the changes can be explained on the basis of alterations in blood volume that are known to occur as the result of pregnancy and of vitamin B_6 deficiency (Brown and Pike, *Fed. Proc.* **17,** *472 (1958)*). Hemoconcentration occurs in deficient, nonpregnant animals. Hemodilution occurs in pregnant animals but is less marked in deficient than in supplemented animals. Thus the increases in total plasma protein and hematocrit and hemoglobin in the deficient, nonpregnant animals probably reflect a reduced blood volume.

Changes in plasma protein in the pregnant, deficient and supplemented rats also probably reflect to a large extent changes in blood volume. This is consistent with the findings of others that vitamin B_6 deficiency does not affect the level of serum proteins or blood amino nitrogen (J. R. Beaton *et al., J. Biol. Chem.* **200,** *715 (1953)*).

However, continued deficiency apparently results in a decrease in hemoglobin and hematocrit, which is not explicable on the basis of blood volume changes and which is potentiated by pregnancy. The authors believe that this indicates a loss of ability to synthesize hemoglobin and red blood cells. To corroborate this they have shown, as have other workers (A. Kornberg, H. Tabor and W. H. Sebrell, *Am. J. Physiol.* **143,** *434 (1945)*; W. W. Hawkins, B. Lechow, and M. K. Evans, *Ibid.* **170,** *155 (1952)*), that the deficient animal is unable to adequately replace red cells lost by removal of blood.

Red blood cell counts and appearance in smears were not reported and, therefore, although hemoglobin and hematocrit levels are known, it is not possible to say whether the altered production of cells is reflected in cells of abnormal size and/or abnormal hemoglobin content. Such values would be desirable and are of such simplicity that they should be included in all studies concerning the red cell.

Others (J. M. Batchen *et al., Brit. J. Nutrition* **2,** *14 (1948)*) have reported that vitamin B_6 deficiency in the rat results in increased red cell count, decreased hemoglobin, reduction in mean corpuscular volume, and reduction in mean corpuscular hemoglobin concentration; that is, a microcytic, hypochromic anemia. Increased numbers of normoblasts also appear. Thus it appears that vitamin B_6 deficiency causes impaired production of normal erythrocytes and this effect may be further potentiated by the demands of pregnancy.

The pathogenesis of the anemia of vitamin B_6 deficiency is not known and future studies might well pursue this objective. Occurring concomitantly with the microcytic, hypochromic anemia is a progressive hypersideremia and storage of iron in tissues as hemosiderin (*Nutrition Reviews* **3,** *29, 343* (*1945*)). There is no evidence of hemolysis and the primary abnormality appears to be a defect in heme synthesis (M. P. Schulman and D. A. Reichert, *J. Biol. Chem.* **226,** *181* (*1957*)), perhaps in the formation of protoporphyrin (G. E. Cartwright and M. M. Wintrobe, *Ibid.* **172,** *557* (*1948*)).

The reason for the development of increased plasma iron is apparently a normal or increased absorption of iron combined with a decreased iron utilization in the formation of hemoglobin. If dietary iron is concomitantly restricted, hyperferremia does not develop (*Nutrition Reviews* **3,** *269* (*1945*); **8,** *5* (*1950*)). Histologic studies of bone marrow in the rat have not been found, but in the pig and dog, in which species the anemia is more severe, there is a marked hyperplasia of erythropoietic elements (*Ibid.* **3,** *29, 343* (*1945*)).

It is interesting that several cases of hypochromic, hypersideremic anemia in man which respond favorably to vitamin B_6 therapy have recently been reported (G. Gehrmann, *Folia Haemat.* **2,** *225* (*1958*); S. E. Snyderman *et al.*, *Am. J. Clin. Nutrition* **1,** *200* (*1953*); J. W. Harris *et al.*, *Proc. Soc Exp. Biol. Med.* **91,** *427* (*1956*)). The relation of this to the hypochromic, hypersideremic anemia in man in which a morphological abnormality of iron accumulation in mitochondria of immature red cells has recently been observed (A. Policard and M. Bessis, *Comptes Rendus Acad. Sci.* **246,** *3194* (*1958*)) is unknown, but it is probable that study by electron microscopy of erythropoietic tissue in vitamin B_6-deficient experimental animals would give valuable information.

The effect of maternal deficiency on the developing embryo is of great interest and is only briefly mentioned in the studies reported here. Marked reproductive upsets in rats depleted of vitamin B_6 prior to pregnancy and continued on a deficient diet during pregnancy have been reported (*Nutrition Reviews* **15,** *20* (*1957*)). These reproductive derangements have consisted of fetal resorptions, failure of implantation and underweight fetuses. No congenital malformations have been reported.

The effect of pyridoxine deficiency on pregnancy is similar to the effect of deficiencies of several other vitamins and essential food factors (M. M. Nelson and H. M. Evans, *J. Nutrition* **31,** *497* (*1946*)), including deficiency of essential fatty acids (G. O. Burr and M. M. Burr, *J. Biol. Chem.* **86,** *587* (*1930*)). The latter relationship is of particular interest because of the apparent importance of vitamin B_6 in essential fatty acid metabolism. It would be of interest to know whether supplementation of the vitamin B_6-deficient diet with a superabundance of essential fatty acids would partially or completely inhibit the effect of vitamin B_6 deficiency on reproduction.

The defects in protein metabolism occurring in vitamin B_6 deficiency are probably responsible for the aberrations in red cell production and fetal development as well as for other abnormalities. The ultimate defect appears to be an abnormality in the metabolism of amino acids so that there is increased amino acid catabolism (E. W. McHenry, *in Current Research of Vitamins in Trophology, p. 21. National Vitamin Foundation, Inc., New York* (*1953*)). Observations consistent with this viewpoint are decreased nitrogen retention (Beaton *et al.*, *loc. cit.*), elevated fasting blood urea (Hawkins, M. L. McFarland, and McHenry, *J. Biol. Chem.* **166,** *223* (*1946*)), increased rate of urea formation in liver slices from deficient rats (E. F. Caldwell and McHenry, *Arch.*

Biochem. Biophys. **48,** *50* (*1954*)), and aggravation of vitamin B_6 deficiency by high protein diets (E. C. Miller and C. A. Baumann, *J. Biol. Chem.* **157,** *551* (*1945*)) and by growth hormone (J. L. Beare *et al.*, *Endocrinology* **55,** *40* (*1954*)).

CECAL ENLARGEMENT IN GERM-FREE ANIMALS

Germ-free animals have ceca which, with their contents, weigh about six times that of conventional animals. This increase in size is apparent in rats by the second week of life.

One of the major difficulties confronting workers in the germ-free area is the development of an enlarged cecum in all species of animals successfully raised in the germ-free state. This situation was recognized as early as 1896 by G. H. F. Nuttal and H. Thierfelder (*Ztschr. Physiol. Chem.* **22,** *62* (*1896*)), who reported that at two weeks of age their germ-free guinea pigs had enlarged ceca filled with a fluid-like material.

B. Wostmann and E. Bruckner-Kardoss (*Am. J. Physiol.* **197,** *1345* (*1959*)) reported that rats and mice raised in the germ-free state could successfully reproduce and rear young even though their ceca were approximately five times normal size. A similar situation in the rabbit or guinea pig, however, appears to have an adverse influence on reproduction. The above investigators report that there has been only one litter of germ-free guinea pigs born to a germ-free mother.

The relationship of the enlarged cecum to reproductive failure is unknown. It has been observed that viable spermatozoa could be secured from epidydimus and ductus deferens of male guinea pigs (B. Philips, P. Wolfe and H. A. Gordon, *Ann. N. Y. Acad. Sci.* **78,** *183* (*1959*)). The ovaries in the females showed no evidence of ovulation or of corpus luteal tissue.

In germ-free rabbits the cecum and its contents may sometimes represent one-third of the body weight. This enlarged cecum sometimes gets twisted, thus preventing cecal flow. This has been the only apparent cause of death in a number of animals (Wostmann and Bruckner-Kardoss, *loc. cit.*).

Wostmann and Bruckner-Kardoss (*loc. cit.*) measured the size of ceca in germ-free rats. They hoped to determine whether the cecal enlargement was due to a deficiency or imbalance of nutrients in the sterilized diets or to the absence of microbes in the intestinal tract. Twenty-one germ-free animals were permitted nothing but milk from the time they were born. After the first ten days they were separated from their dam but every four hours the mother was placed in their cage for a two-hour nursing period. By this means the young were kept away from solid food. Another 21 germ-free animals were permitted access to a sterilized semi-purified diet in addition to the mother's milk. Conventional control animals were reared in a regular animal colony.

At the seventh day of life one-third of the animals in each group was sacrified, but no difference was found in the body weights of the animals nor in the size of their ceca. However, by the sixteenth day the seven germ-free rats with access to the diet weighed 23 g. and had ceca averaging 1.6 g., while the conventional rats weighed 33 g. with ceca weighing 0.4 g. The germ-free rats receiving nothing but mother's milk weighed 20 g. and had ceca weighing 0.9 g., while the conventional control rats receiving nothing but mother's milk weighed 24 g. and had ceca weighing 0.3 g. Similar differences between the germ-free and the conventional animals were apparent at the twenty-fifth day, at which time the weights of the ceca of the germ-free animals were from 5.5 to 6.4 times those in the conventional rats.

Five male and four female germ-free rats were continued on the sterile semi-purified diet until they were 42 days of age. At this time they weighed 94 and 74 g., respectively, while the conventional animals weighed 159 and 119 g., respectively. The weight of the ceca in the germ-free animals was from 6.6 to 7.4 times that in the conventional animals. Similar results were secured using a sterilized "practical type" diet.

The investigators assumed that mother's milk is a fairly complete food. Thus when the animals receiving nothing but mother's milk showed enlargement of the ceca, they suggested that this enlargement was due to some factor other than nutritional deficiency.

In the present study, the germ-free rats did not grow as rapidly as the conventional animals (94 g. *vs.* 159 g. for males at 42 days of age). This was contrary however, to earlier observations on chicks by the same authors (*Nutrition Reviews* **17,** *236 (1959)*). Thus until further study has been made of the poor growth of the germ-free rat, it may be premature to conclude that the enlarged ceca is not due to a nutritional deficiency.

The incidental observations made in conjunction with the above study indicated that the water content of the small intestine was the same in the germ-free and the conventional rats (76 *vs.* 74 per cent). On the other hand, the ceca of the germ-free rats contained considerably more water than in the conventional animals (84 *vs.* 74 per cent). To determine whether the bacterial flora in the conventional rats might bind minerals and thus reduce the osmotic pressure, Wostmann and Bruckner-Kardoss fed germ-free rats a purified diet containing a very low salt content. This, however, had no effect on the enlargement of the ceca.

They also observed that the histamine concentration in the cecal contents of germ-free guinea pigs was 2.5 μg. per g. whereas in the conventional animals it was 10.3 μg. per g. Rats showed similar but smaller differences. The authors were unable to increase the histamine level in the ceca of germ-free rats by anal catheterization.

The above observations are intriguing in that they show another marked difference in the physiology and anatomy of the germ-free and conventional animal. One might hope that subsequent work will utilize a sterile extract of the intestinal contents of conventional rats. If this or a sonic preparation of the intestinal contents were fed to germ-free animals, it should be possible to determine whether bacterial products are responsible for the smaller ceca in the conventional animals.

EXPOSURE OF NIACIN AND AMINO ACIDS TO ETHYLENE OXIDE

Exposure of certain nutrients to ethylene oxide results in hydroxyethylation of tertiary nitrogen atoms and of sulfur atoms with consequent loss of biological activity.

A decrease in the nutritional value of food exposed to the fumigant, ethylene oxide, was demonstrated a few years ago by E. A. Hawk and O. Mickelsen (*Science* **121,** *442 (1955)*; *Nutrition Reviews* **14,** *55 (1956)*). These workers established that thiamine was one of the nutrients adversely affected by ethylene oxide, but concluded that other factors must have been altered as well.

Since then, treatment with ethylene oxide has been shown to cause at least partial destruction of the following vitamins: riboflavin, niacin, pyridoxine, and folacin (H. Bakerman *et al., J. Agr. Food Chem.* **4,** *956 (1956)*); and the following amino acids: histidine, methionine (H. G. Windmueller, C. J. Ackerman and R. W. Engel, *J. Nutrition* **60,** *527 (1956)*) and, most recently, lysine (Windmueller, Ackerman and Engel, *J. Biol. Chem.* **234,** *895 (1959)*). The general

problem of chemicals and food processing has been reviewed by Mickelsen (*Nutrition Reviews* **15,** *129* (*1957*)).

Until recently, the nature of the reaction of ethylene oxide with these nutrients had not been elucidated. However, Windmueller, Ackerman, Bakerman and Mickelsen (*J. Biol. Chem.* **234,** *889* (*1959*)) have now identified reaction products of ethylene oxide with niacinamide and with niacin, and Windmueller, Ackerman and Engel. (*Ibid.* **234,** *895* (*1959*)) have strong evidence of the nature of the reaction products with histidine, methionine and cysteine.

In the first study, a solution of the pure nutrient in a previously evacuated vessel was exposed to ethylene oxide gas at approximately atmospheric pressure. Facilities were available to measure and regulate pH during the exposure, since at times reaction products were strongly alkaline. It was properly realized that the conditions used did not duplicate commercial methods of ethylene oxide sterilization of foods. However, the objective was to produce large quantities of reaction products for identification.

When the pH of the reaction mixture of niacinamide and ethylene oxide was maintained between 7 and 10.5 by adding hydrochloric acid, a product was obtained in crystalline form, which was identified as the chloride of N′-(2-hydroxyethyl) niacinamide. In other words, the hydroxyethyl group had added to the tertiary ring nitrogen of niacinamide. Since this product fluoresced when subjected to the assay procedure of J. W. Huff and W. A. Perlzweig (*J. Biol. Chem.* **167,** *157* (*1947*)) for N′-methylnicotinamide, a fluorometric assay was used to quantitatively determine the product. The N′-(2-hydroxyethyl) nicotinamide was unstable at pH 11 to 12, producing a variety of reaction products, and this explained why it was not possible to identify this reaction product unless the highly alkaline conditions were avoided.

The authors present evidence that this same compound is probably produced in foods treated with ethylene oxide. N′-(2-hydroxyethyl) nicotinamide neither replaced nor was a metabolic antagonist for niacin as judged by the response of the microorganism, *Lactobacillus arabinosus*, or of chicks maintained on low-niacin regimens.

A similar reaction occurred between ethylene oxide and niacin, and the product was identified as the internal salt, the betaine of N′-(2-hydroxyethyl) niacin. At pH 6 or above, the biological activity of niacin was destroyed to a greater extent than that of niacinamide. The destruction of both compounds by ethylene oxide was highly dependent on temperature and was more extensive at pH values above neutrality.

Of interest was the discovery that DPN, a coenzyme form of niacin, was completely stable to ethylene oxide treatment as determined by microbiological assay. This fact, along with variation in moisture, pH and reaction temperature, probably largely accounts for the marked variations in susceptibility to ethylene oxide fumigation of niacin activity in various food products. For example, under standardized conditions of fumigation, only 22 per cent of the niacin activity was destroyed in brewers' yeast compared to 84 per cent in enriched white flour.

Windmueller and co-workers point out that each of the vitamins shown to be labile to ethylene oxide treatment contains a tertiary heterocyclic nitrogen, and, although direct evidence is lacking, the authors suggest that this site is most likely the point of attack by ethylene oxide.

In the second paper, Windmueller *et al.* (*J. Biol. Chem.* **234,** *895* (*1959*)) present results of a study of the reaction products of ethylene oxide with proteins and with certain amino acids. While previous work had shown that casein treated with ethylene oxide exhibited a lowered biological availability of histidine and methionine, the recent

work indicates that lysine is also affected and that lactalbumin and egg albumin behave similarly to casein.

In this study, imidazole was used as a model compound and comparisons were made of the reaction of ethylene oxide with histidine and with imidazole. From the results it was concluded that ethylene oxide caused hydroxyethylation of both nitrogen atoms of the imidazole ring. Destruction of histidine in intact protein (casein) exposed to ethylene oxide was demonstrated by chemical assay for histidine and was shown to be increased markedly if the protein contained moisture. However, in certain proteins (chymotrypsin) the histidine moieties appeared not to react unless the protein was denatured in 8 molar urea.

Chemical studies of the reaction products of ethylene oxide with methionine and with N-acetylmethionine indicated that one hydroxyethyl group had added to the sulfur atom of these compounds to produce a sulfonium group. A similar double alkylation of the mercapto group of cysteine occurred and with this amino acid the primary amino group also became alkylated. Although the reaction product with lysine was not identified, it was assumed that hydroxyethylation of one or both of the primary amino groups of lysine could account for the decreased availability of this amino acid.

The authors generalize by stating that the reactions of these nutrients with ethylene oxide under mild conditions involve electrophilic hydroxyethylation of an atom with one or more lone pairs of electrons, either nitrogen or sulfur. In no case has it been shown that these hydroxyethylated derivatives possess the biological activity of the original nutrient. These studies amply clarify in a qualitative manner how ethylene oxide, as a commercial fumigant, may lower the nutritive value of foods.

ENHANCEMENT OF CALCIUM ABSORPTION BY CARBOHYDRATES

Studies of Ca^{45} absorption from the upper and lower small bowel of rats indicate that several sugars enhance calcium absorption in the ileum. Simple sugars are absorbed too rapidly to contribute to this effect.

In economically depressed countries, the diet of most persons is composed more of vegetable foods than of animal foods. Usually these circumstances result in a lower intake of calcium than generally considered optimal. Nevertheless, A. R. P. Walker *Am. J. Clin. Nutrition* **3,** *114* (*1955*)) reported that children in several such countries did not have a high incidence of rickets, nor was their rate of growth benefited by the addition of calcium to their diets. A similar report (*Nutrition Reviews* **13,** *131* (*1955*)) gave evidence that children who had eaten a diet composed largely of cereals apparently were not deficient in calcium since the addition of milk or of fortified flour to their diets failed to improve their growth. Either our estimates for calcium requirement have been too high, or these children have been able to use calcium more economically than children fed the usual American diet.

For many years it has been recognized that dietary lactose facilitates absorption of calcium from the gut. This subject was reviewed recently (*Nutrition Reviews* **18,** *115* (*1960*)) and evidence was presented to show that lactose has a direct effect on the intestinal wall.

O. W. Vaughan and L. J. Filer, Jr., (*J. Nutrition* **71,** *10* (*1960*)) studied this problem in further detail. They considered several theories: (1) that lactose lowers the pH of the gut by promoting fermentation, (2) that some sugars influence the bone cells in the process of ossification, (3) that lactose (as well as other sugars) may cause stimulation of the flow of digestive juices, including

bile, which in turn may augment calcium absorption.

The authors employed Ca^{45} to study the rate of absorption from isolated loops of bowel in rats. Loops were isolated in the proximal small bowel (duodenum) or in the distal small bowel (ileum). Test solutions containing one of the sugars and calcium chloride tagged with Ca^{45} were injected into these intestinal loops. The effects of bile and of other ions (Mg^{++}, Sr^{++}, Na^{+}) were similarly studied. After four hours, the animals were sacrificed and their femurs ashed for determination of Ca^{45} content. In some of the studies, the intestinal loops also were removed to determine the amount of Ca^{45} remaining unabsorbed. The sugars employed were lactose, sorbitol, glucose, sucrose, fructose, galactose and xylose.

None of the substances had a significant enhancing effect on the rate of calcium absorption from the duodenum. However, in the ileum each of the sugars enhanced the rate of Ca^{45} absorption. The addition of Mg^{++} or Sr^{++} to the test solution prevented the enhancing effect of sugars, but Na^{+} did not. Addition of phosphate inhibited calcium absorption both alone and with sugar.

It is apparent that most of the calcium was absorbed from the distal portion of the bowel. Although all of the sugars tested could enhance this rate of absorption, simple sugars would not reach this level but would be absorbed in the upper bowel. Indeed, it has been shown that feeding rapidly absorbed sugars with a test dose of calcium had no effect. However, the more complex carbohydrates, which are absorbed lower in the digestive tract, did enhance absorption of calcium (R. H. Wasserman and C. L. Comar, *Proc. Soc. Exp. Biol. Med.* **101,** *314 (1959)*).

This may explain in part the ability of children eating crude vegetable diets to absorb a quantity of calcium sufficient for growth, even though the supply is meager. In all probability other factors may be involved, but complex carbohydrates are undoubtedly of assistance.

CHANGES OCCURRING WITH VITAMIN A DEFICIENCY

With increasing duration of vitamin A deficiency in calves, cerebrospinal pressure increases as a result of increased volume. A decrease in potassium level of aqueous humor also occurs.

In contrast with the definite changes in epithelial tissues, bones and teeth, which occur in avitaminosis A, the metabolic role of vitamin A aside from its function in vision remains obscure. Observations of L. A. Moore and J. F. Sykes (*Am. J. Physiol.* **130,** *684 (1940)*) that cerebrospinal fluid pressure increased in vitamin A-deficient calves soon led to similar observations in other species. Furthermore, the similarity of the mechanism whereby cerebrospinal fluid and aqueous humor are formed (H. Davson, *Physiology of Ocular and Cerebrospinal Fluids. Little Brown and Co., Boston (1956)*) has raised the suggestion that intraocular pressure may increase in vitamin A deficiency.

Some of these considerations led B. A. Dehority *et al.* (*J. Dairy Sci.* **43,** *630 (1960)*) to study the changes occurring in the composition of cerebrospinal fluid and aqueous humor with increasing duration of vitamin A deficiency in an attempt to relate these changes to the increased pressures of these fluids in the deficient animal.

Dehority and co-workers used male Holstein calves, which they depleted to a plasma vitamin A value of 8.3 μg. per 100 ml. of plasma and then supplemented with carotene (supplied by alfalfa) at levels of 16, 24, 32 and 40 μg. per pound of live weight

per day. The lower two levels were designed to provide marked increases in cerebrospinal fluid pressure, the 32 μg. level to produce moderate increases, and the 40 μg. level to produce no increases in the pressure of this fluid. The calves were maintained on these levels of carotene intake for a period of 16 weeks, at which time the animals were sacrificed and extensive analyses carried out on various tissues and fluids of the body. In addition to the analyses, cerebrospinal fluid pressures were measured ten and three days prior to sacrifice. Intraocular pressures were measured before carotene supplementation was begun and five days prior to sacrifice.

Cerebrospinal pressures increased from 44 ml. of saline with the carotene intake of 40 μg. per pound of live weight per day to 175 ml. with the 24 μg. intake. Pressure with the 32 μg. intake was 131 ml. In an effort to determine the mechanisms whereby this pressure was caused, these investigators point out that in the vitamin A deficiency pattern where tissue changes were not apparent, chemical changes in blood serum, cerebrospinal fluid and aqueous humor were for the most part nonsignificant. Plasma vitamin A showed increases with increasing levels of carotene intake and there was also an increase in carotenoid and vitamin A content of the liver with increasing levels of carotene.

The most outstanding observation was the very little change in composition of the cerebrospinal fluid. Thus these investigators concluded that the pressure associated with vitamin A deficiency is due to an increased volume of cerebrospinal fluid caused either by increased formation of the fluid or by decreased absorption, or possibly both. An evaluation of the aqueous humor showed that the slight increase in intraocular pressure was not significant and it was concluded that either the method of measurement was of insufficient accuracy or that there were factors other than vitamin A deficiency controlling the regulation of intraocular pressure.

In the chemical evaluation of the constituents, it was found that the potassium level of aqueous humor showed a significant decrease with the lower intakes of carotene. This was accompanied by an increase in chloride content with a slight net increase in osmotic pressure.

The investigations of Dehority and coworkers are of interest since they emphasize the very slight alterations in the chemical composition of fluids which nonetheless exhibit changes in volume and, in the case of the cerebrospinal fluid, changes in pressure. The changes occurring in the aqueous humor certainly are of interest and point to the need for further study of such alterations under more severe conditions of vitamin A deficiency. The very suggestive work of these investigators points up the need for continued study of the locations of cerebrospinal fluid absorption and formation as possible areas where vitamin A exerts a specific metabolic function.

NOTES

Site of Fatty Acid Absorption

The intestinal absorption of fat has been investigated so frequently during the past two decades that at times it has seemed that little could be gained from its further study. Nevertheless, although the mechanism of absorption in rats is now fairly well known, it is probable that species differences exist. Moreover, certain other details remain to be worked out, such as the site of absorption.

In order to clarify this phase of fat absorption, J. M. Johnson (*Proc. Soc. Exp. Biol. Med.* **100,** *669 (1959)*) made use of a technique which he had developed previously (*Ibid.* **98,** *839 (1958)*) to study fatty acid absorption. Small everted sacs were prepared from successive segments of the small intestines of hamsters. These were filled with bicarbonate buffer and glucose solution and were immersed in solutions of palmitic-1-C^{14} acid-albumin complex also containing glucose and were incubated with 95 per cent oxygen and 5 per cent carbon dioxide at 37°C. Aliquots of serosal and mucosal solutions and homogenized intestinal wall were plated for counting and the lipids were separated into free and esterified fatty acids.

The results of these analyses revealed that activity in the serosal solutions and intestinal walls was greatest for the uppermost jejunal segment and decreased progressively with each lower segment. The reverse was true for the mucosal solutions. This information was strengthened by the finding that, generally, the ratios of activity of the esterified to free fatty acids of the mucosal portions were greater in the upper than in the lower segments. In the serosal solutions, the activity of the esterified portion of the fatty acids was greater than 90 per cent of the total.

These results indicate a considerably greater ability of the upper portion of the hamster intestine to absorb and esterify fatty acids. The actual site of maximum absorption, however, may depend partially on other factors such as size of fat meal, intestinal motility and state of emulsification. Thus, J. A. Benson, G. N. Chandler, F. E. Vansteenhuyse and J. O. Gagnon (*Gastroenterology* **30,** *53 (1956)*; *Nutrition Reviews* **15,** *93 (1957)*) reported that I^{131}-labeled olive oil is absorbed maximally in the third quarter of the small intestine of the rat. If rats and hamsters can be compared and if iodinated olive oil and fatty acid-albumin complex are absorbed by the same mechanism, their reasoning that this portion of the intestine has "a heightened capacity for cell work, perhaps by cellular enzyme systems," is not supported by the experiments of Johnson.

It seems evident from these considerations that even in the case of this relatively simple phase of fat absorption, considerable work remains to be done.

Extreme Hypocholesterolemia with Steatorrhea

In the voluminous literature on cholesterol metabolism, much of it relating to hypercholesterolemia and its presumed causes and effects, there are far-reaching assumptions as to the value of reducing serum cholesterol levels in the prevention of atherosclerosis. Scarely anything is written, however, about hypocholesterolemia.

I. S. Friedman, H. Cohn, M. Zymaris and M. G. Goldner have reported a case of lifelong "Hypocholesteremia in idiopathic steatorrhea" under this title (*A. M. A. Arch. Int. Med.* **105,** *112 (1960)*). Their patient, a 36-year-old white man, has been confined to a hospital for chronic diseases for over 20 years; throughout this period his serum total cholesterol concentration has ranged between 25 and 45 mg. per cent.

The clinical history and findings are exceedingly complex. Celiac disease was diag-

nosed at 18 months and treated with a banana and meat diet for years. When 14 years old, the blood serum cholesterol level was found to be 42 mg. per cent. At 36 years of age the patient is malnourished. In the laboratory tests performed over the years, occasional traces of protein were found in the urine. The hemoglobin remained near 14 g. per cent, red blood cells over 4 million per cubic millimeter, plasma albumin 4.3 g. per cent, and globulin 2.1. Cephalin flocculation at times varied from + to +++; serum amylase was somewhat depressed (29 to 53 units per cent).

The outstanding chemical abnormalities were in the serum lipids, which totaled only 110 mg. per cent; of this, total cholesterol was 25 mg. per cent (7 free, 18 esterified). Vitamin A absorption was close to zero. Glucose tolerance was normal. Creatinine excretion was above normal, probably because of muscle wasting. The 24-hour urinary excretion of 17-ketosteroids was very low (5.5 mg.) and there was no increase after corticotrophin injection. Both fat digestion and absorption were shown to be impaired.

Normal thyroid and liver function tests exonerated these organs. However, the low urinary excretion of 17-ketosteroids and its failure to rise after corticotrophin injection suggested hypofunction of the adrenal cortex as a factor in the patients low serum lipid values.

The infantile sprue and later steatorrhea in this case surely represent one entity, and the authors' diagnosis is nontropical sprue or adult celiac disease, in spite of the normal glucose tolerance, lack of anemia and normal serum calcium. The patient's diet is self-selected: 2400 calories, high in carbohydrate, with much bread, moderate protein and little fat. He has refused a gluten-free diet. A number of physical findings suggest genetic disease.

Because of the normal cholesterol and phospholipid content of the stools, Friedman *et al.* infer that malabsorption of these substances is not responsible for their low serum levels. The evidence does not establish that the patient has either decreased formation or increased degradation of his serum lipids.

As to the diminished adrenal cortical function, one is unable to relate it in detail to the other findings inasmuch as hypocholesterolemia may be both cause and effect of decreased steroid output. One can believe, with the authors, that the adrenal cortical deficiency is the result of malnutrition, but this does not say much as to the relationship between adrenal steroids, lipid absorption and serum lipid levels. Established vitamin deficiencies in this patient complicate matters still further.

Although moderate hypocholesterolemia occurs in cases of steatorrhea, its relation, in this patient, to adult celiac disease, to adrenal cortical hypofunction, or to a possible genetic biochemical defect of lipid metabolism remains conjectural. Withal, the value of this case report is to establish that a man has existed for at least 20 years with a serum cholesterol concentration of 25 to 45 mg. per cent.

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